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Full Research Article

Effect of Acute Iron Toxicity on Key Antioxidative Enzymes in Contrasting Rice (*Oryza sativa* L.) Cultivars of North-East India

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Abstract

Iron toxicity is a nutritional disorder frequently occurring in lowland soils where rice is cultivated, due to the formation of excess ferrous iron in reduced soil condition. To contribute in understanding the mechanisms involved in response to excess iron, an investigation was carried out to determine the variation in symptom expression in leaves, tissue iron distribution and antioxidative enzyme activity in five rice cultivars viz., Kanatara, Katakchara, Maibetikala, Chinari and Lalgura of Northeast India. Hydroponically grown rice seedlings of these cultivars were exposed to a high Fe concentration (1000 mg L^{-1}= 17.9 mM Fe^{2+}), harvested after five weeks growth to study the antioxidative responses to excess iron stress. The iron accumulated in different plant tissues were analysed and the relatively tolerant cultivars showed higher retention of iron in root tissues, confirming that the dominant tolerance mechanisms in the tolerant cultivars were related to root based mechanism (i.e., iron exclusion). Higher superoxide dismutase and catalase activities were observed in cultivars Kanatara, Katakchara, Chinari and Lalgura. Conversely, cultivar Maibetikala showed significant reductions in superoxide dismutase and catalase activities. In response to high iron level in growth medium, cultivar Maibetikala maintained higher ascorbic peroxidase and glutathione reductase activities. Results of the study reveal that the rice cultivars differ in their response to iron toxicity and the tolerance attributes largely depend on the enhancement of antioxidative enzymes, suggesting that antioxidative enzymes has a significant role in protecting the plants against oxidative damage under iron toxic conditions.

1. Introduction

Rice is the staple food crop in the North-East India, cultivated over 3.51 million hectares, however, the average productivity of North-East Region remains very low as compared to national average. One of the major factors contributing to low productivity is the soil acidity (pH<5.5), leading to severe deficiencies of phosphorus with toxicities of iron and aluminium in plants (Dohling, 2011). Iron is an essential element in plants which plays an important role in many physiological processes such as chloroplast development, chloroplast biosynthesis, ribonucleotide and dinitrogen reduction, as well as energy-yielding electron transfer reactions of respiration and photosynthesis (Hell and Stephan, 2003); however, excess concentrations of ferrous (Fe^{2+}) iron may lead to Fe toxicity. Iron toxicity is reported to be the most widespread nutritional disorder affecting the rice production in acid soils (Finatto et al., 2015).The excessive uptake of ferrous iron by plants and its xylem transport *via* transpiration stream into the leaves can lead to the generation of reactive oxygen species (ROS) (Thongbai and Goodman, 2000) cause an irreversible damage to membrane lipids, proteins and nucleic acid (Stein et al., 2009a; Nagajyoti et al., 2010). Subsequently, they oxidise chlorophyll and reduce leaf photosynthesis (Pereira et al., 2013) entailing yield losses in the range of 10-100% (Audebert and Fofana, 2009).

Plant cells employ a defensive pathway to detoxify the free radicals to protect the tissues from oxidative damage, through enhancement of antioxidative mechanisms (Majerus et al., 2007b). Therefore, tolerance of plants to excess iron may be improved if the free radicals are scavenged by an enhanced antioxidative defence system. Previous reports on antioxidative response of plants in response to iron stress indicate that rice

cultivars differ in their response to iron tolerance and tolerance properties depend largely on the enhanced activity of these enzymes (Saikia and Baruah, 2013). In context of the rich bio-diversity of the region, it is appropriate to screen rice genotype for tolerance to Fe toxicity as an effective approach for soil amelioration against Fe toxicity.

The present study was undertaken to screen the indigenous rice cultivars of the region, for tolerance to excess iron stress, and further put under investigation to assess the contribution of the antioxidative mechanism in the long term response of rice to a high Fe^{2+} dose using selected cultivars viz., Katakchara, Kanatara, Chinari, Lalgura and Maibetikala, which were screened from a group of twelve rice cultivars.

2. Materials and Methods

2.1. Planting material

Five tall indica rice cultivars viz., Katakchara, Kanatara, Chinari, Lalgura and Maibetikala obtained from germplasm collection of the ICAR-RC-NEH Region, Tripura Centre, India were selected to study the antioxidative responses exposed to excess Fe stress of 1,000 mg L^{-1} Fe^{2+} as Fe_2SO_4. $7H_2O$ (Wu et al., 2014). Experiments were conducted in Plant growth chamber (Conviron model no. PGW40) facilities of the institute following standard methods (Wu et al., 2014) with the day/night temperature set at 30/25 °C, photosynthetically active radiation (PAR) light of 400 µmol m^{-2} sec^{-1}. Rice seeds were soaked in demineralized water and germinated at 30 °C in the dark for 72 hours. Subsequently, germinating seeds were floated in 70.5 mg L^{-1} $CaCl_2$ and 1.625 mg L^{-1} $FeCl_3$ solution in light for another 5 days. Homogenous seedlings were selected and transplanted into 40 L tanks filled with half strength Yoshida nutrient solution. In all experiments, three replicate plants $rice^{-1}$ cultivars were used. The pH value during the experimental period was maintained at 5.5 and solutions were exchanged every 10 days. Five weeks after the plant growth, half of the plants were exposed to a excess iron stress of 1000 mg L^{-1} for 5 days. To maintain the low redox potential in the solution, N_2 gas was percolated into the culture solutions for 15 minutes every 2 hours. Leaf bronzing scores were measured on the three youngest fully expanded leaves of the main tiller after five days. Plant materials were harvested for Fe distribution and antioxidative enzyme study. Plant shoots of contrasting lines were oven-dried at 60 °C until the weight was constant and ground to a fine powder. Fe concentrations in shoots were determined after digesting 250 mg of dry samples with 4 ml 65% HNO_3 at 180 °C for 8 hours followed by dilution to 25 ml and filtration. Standard and sample solutions were measured using atomic absorption spectroscopy (AAS, GBC). All data were analyzed using SPSS 16.0 for windows. One-way analysis of variance (One-way ANOVA) was used to determine whether any significant variation existed between the treatments. When overall differences were found, differences between means were tested by Duncan multiple range test. All differences were considered significant at 5% and the results are presented as mean±S.E. (standard error).

2.2. Analysis of antioxidant enzymes

2.2.1. Superoxide dismutase (SOD)

0.5 g of the leaf samples were homogenized in chilled extraction buffer (pH 7.5) which contains 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.4 mM EDTA, 5 mM $MgCl_2$, 10 % glycerol, 1% polyvinylpyrrolidone (PVP), 2 mM dithiothreitol (DTT), and 1 mM phenylmethane sulfonyl fluoride (Gregenheimer, 1990). The homogenate was then centrifuged at 14,000×g at 4 °C for 20 min. The supernatant was used for enzyme activity assays and stored in -80 °C for further processing. SOD activity was assayed according to the method of (Gupta et al., 2009). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), containing 0.2 mM EDTA, 57 µM nitroblue tetrazolium (NBT), 1.13 µM riboflavin, 0.025 % Triton X-100, and enzyme in a total volume of 1 ml. The superoxide formation was recorded at 560 nm using a UV-vis spectrophotometer. One unit of SOD activity was expressed as the amount of enzyme required to cause 50% inhibition of H_2O_2 oxidation under the experimental conditions.

2.2.2. Catalase (CAT)

Leaf samples (0.5 g) were homogenized in chilled extraction buffer (pH 7.5) containing 50 mM HEPES, 0.4 mM EDTA, 5 mM $MgCl_2$, 10 % glycerol, 1 % PVP, 2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (Gregenheimer, 1990). The homogenate was then centrifuged (12,000 rpm) at 4 °C for 20 min. The supernatant was used for enzyme activity assays and also stored in -80 °C for further processing. CAT activity was determined spectrophotometrically by measuring the rate of H_2O_2 disappearance at 240 nm, taking dA/dt at 240 nm as 43.6 mol/l^{-1} cm^{-1} (Patterson et al., 1984). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 10.5 mmol/l H_2O_2. The reaction was run at 25 °C for 2 min, after adding the enzyme extract containing 20 µg of protein and the initial rate of decrease in absorbance at 240 nm was used to calculate the activity.

2.2.3. Ascorbic peroxidase (APX)

Leaf tissue was ground in isolation buffer (50 mM phosphate buffer pH 6.8, 1 mM ascorbate). The extract was centrifuged at 12,000 rpm for 10 min and the supernatant was taken. The reaction mixture (3 ml) contains 1.5 ml of 0.1 M potassium phosphate buffer pH 6.8, 0.5 ml of 6 mM ascorbate, 0.5 ml of 12 mM H_2O_2, and 0.5 ml of enzyme extract. Change in absorbance was monitored at 290 nm. Extinction coefficient

used was 2.8 mmol/l^{-1} cm^{-1} (Ramel et al., 2009).

2.2.4. Glutathione reductase (GR)

Leaf tissues were ground in 100 mM phosphate buffer (pH 7.6), and the homogenate was centrifuged at 12,000 rpm for 10 min. The supernatant was taken for enzyme assay. GR activity was determined as described by (Foyer et al., 1976). The oxidized glutathione (GSSG)-dependent oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was followed at 340 nm in a 1 ml reaction mixture containing 100 mM sodium phosphate buffer, pH 7.8, 0.5 mM GSSG, 50 µl extract, and 0.1 mM NADPH.

3. Results and Discussion

Ferrous-iron toxicity is one of the widespread nutritional disorders causing various physiological imbalances resulting in yield reduction in rice crops (Majerus et al., 2007b). In the present investigation, differential expression of leaf bronzing symptom was observed among the studied rice cultivars, ranging from 1.0 to 5.0 in response to excess iron stress (Table 1). Among the subjected cultivars, pronounced symptoms were observed in cultivars Maibetikala, whereas the cultivars Lalgura and Chinari expressed mild symptoms. Relatively low leaf bronzing scores were observed in cultivar Katakchara and Kanatara. These observations indicated relatively higher tolerance of cultivars Katakchara, Kanatara, Lalgura and Chinari as compared to cultivar Maibetikala. Symptoms like leaf bronzing are often used as a phenotypic trait for examining abiotic stress including ferrous-iron toxicity. Under Fe toxic conditions, leaf bronzing is strongly correlated with yield reduction in rice (Asch and Becker, 2005) and thus, leaf bronzing score can be considered as a screening trait for Fe toxicity tolerance in rice.

Table 1: Fe^{2+} distribution ($\times 10^3$ mg kg^{-1}) in the tissues of 5 rice cultivars exposed to 2 mg L^{-1} and 1000 mg L^{-1} at 5 week stage of growth in the pot experiment

Rice cultivar	Leaves		Stems		Roots		Symptom score
	2	1000	2	1000	2	1000	
Kanatara	0.27	0.74	0.55	1.05	1.56	2.79	1.5
Maibetikala	0.61	1.08	1.19	1.83	0.41	4.87	5.0
Lalgura	0.18	0.68	0.64	0.82	0.13	1.66	1.0
Katakchara	0.35	0.64	0.44	0.99	2.92	3.58	2.0
Chinari	0.19	0.58	0.26	0.73	1.98	4.97	1.0
SEM	0.12	0.31	0.28	0.26	0.19	0.35	
Prob (diff. in cultivar mean	NS	NS	S	S	S	S	

The leaf tissue Fe^{2+} concentration among the cultivars did not vary significantly both in control and stressed condition. However, the leaf symptom evaluation revealed a higher score of 5 in relatively sensitive cultivar Maibetikala. A possible link between the leaf symptom expression and the chemical transmitted by the root system was earlier suggested by Samaranayake et al., 2012, which may be stronger in the susceptible variety than the resistant one. The highest stem Fe^{2+} concentration was recorded in Maibetikala and the lowest in Chinari in response to excess iron stress (Table 1). A high concentration of iron detected in leaf and stem of Maibetikala suggest possible translocation of iron from stem to leaf tissue, which is in agreement with earlier findings by Onaga et al., 2013, indicating stem as a passive conductor negating its role in inclusion or avoidance mechanism.Significant difference was also observed in root tissue Fe^{2+} concentration among the cultivars in control as well as stressed condition. The highest root Fe^{2+} concentration was recorded in Katakchara and the lowest concentration recorded in roots of Lalgura (Table 1) in response to excess iron stress.Our findings also suggest that the retention power of rice roots plays a major role in inclusion or avoidance mechanism (Nozoe et al., 2008). Despite lower retention of iron in root, cultivar Lalgura showed low Fe uptake in stem and leaf. On the contrary, rice cultivar Maibetikala retained high concentration of iron in roots and showed markedly higher Fe content in stem and leaf tissues. This could possibly explain differential symptom expression observed in leaf of these cultivars as a linear relationship exist between the leaf Fe concentration and leaf symptoms (Onaga et al., 2013). No precise explanation to the difference observed in root retention among the cultivars studied could be brought out as the amount of iron excluded by the roots was not quantified during our investigation.

Lalgura and Chinari cultivars showed significantly ($^*p<0.05$) higher SOD activity among the five cultivars under control condition (Figure 1). In response to excess iron stress, the cultivars recorded a sharp increase in SOD activity except the cultivar Maibetikala (Figure 1). The cultivar Chinari showed highest ($^*p<0.05$) activity among the cultivars subjected to excess iron stress. The CAT activity declined in response to excess iron stress in all the five cultivars. The cultivar Chinari exhibited higher activity pre and post treatment among the cultivars used whereas cultivar Maibetikala showed lowest response pre and post treatment (Figure 2). A gradual increase in APX activity was observed in response to excess iron stress in all the cultivars (Figure 3). The cultivar Kanatara exhibited higher activity pre and post treatment with excess iron among the cultivars used whereas cultivar Maibetikala

showed lowest response pre and post treatment (Figure 3). GR activity also increased in all the cultivars in response to excessiron stress (Figure 4). Cultivar Maibetikala exhibited lowest and highest activity pre and post treatment respectively among the cultivars. Further, the assayed antioxidative enzyme activities were observed to be negatively correlated with the symptom expression in leaf (Table 2). It has been reported that excess Fe treatment increases lipid peroxidation or induces oxidative stress in plant tissues (Majerus et al., 2007b). SOD, CAT, APX and GR are important anti-oxidative enzymes in plant leaves that can protect the plants from ROS damage. It has been earlier demonstrated that excess Fe increased the activities of SOD, APX, CAT and GR (Saikia and Baruah, 2013; Agarwal et al., 2006). In our study, with rice leaves of selected five cultivars showed that excess Fe increased the activities of SOD, APX and GR and decreased the CAT activity except the cultivar Maibetikala where in SOD activity was decreased which may be due to enzyme inhibition resulting from accumulation of greater amount of Fe^{2+} iron in the shoots. Our result supports the earlier findings where the suppression of SOD and CAT activity in sensitive rice cultivars after exposure to excess iron was reported (Saikia and Baruah, 2013). The authors further suggested that a common ion effect mechanism may operate in sensitive rice cultivars; resulting in decreased SOD activity as higher Fe^{2+} concentration may reduce the SOD activity by accelerating

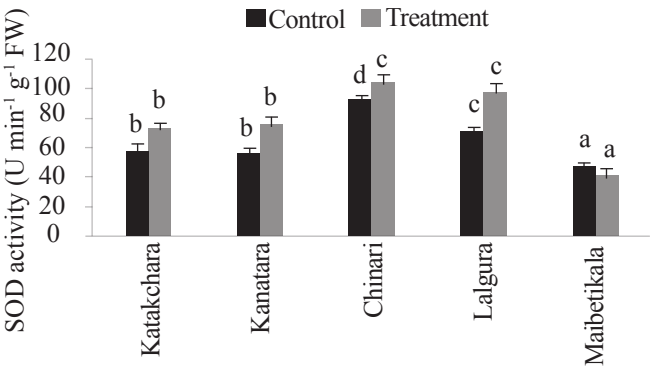

Figure 1: Superoxide dismutase activity in five weeks old rice seedlings treated with 2 and 1000 mg L^{-1} Fe^{2+} in the form of Fe_2 SO_4. $7H_2O$. Each data point is the average of three replicates. The *errorbar* represents SD

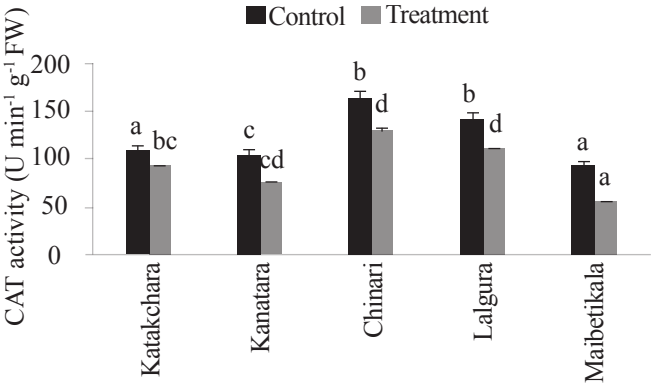

Figure 2: Catalase activity in five weeks old rice seedlings treated with 2 and 1000 mg L^{-1} Fe^{2+} in the form of Fe_2 SO_4. $7H_2O$. Each data point is the average of three replicates. The *error bar* represents SD

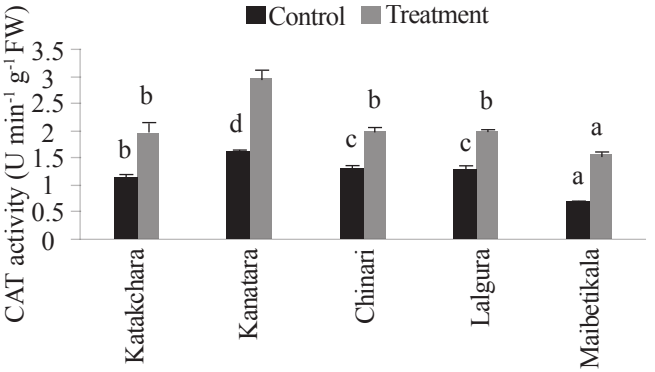

Figure 3: Ascorbic Peroxidase activity in five weeks old rice seedlings treated with 2 and 1000 mg L^{-1} Fe^{2+} in the form of Fe_2 SO_4. $7H_2O$. Each data point is the average of three replicates. The *error bar* represents SD

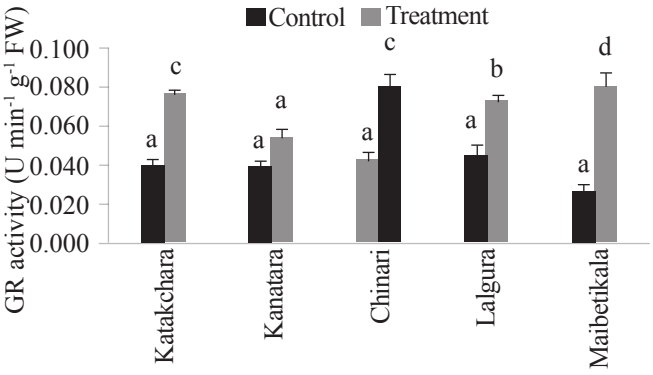

Figure 4: Glutathione reductase activity in five weeks old rice seedlings treated with 2 and 1000 mg L^{-1} Fe^{2+} in the form of Fe_2 SO_4. $7H_2O$. Each data point is the average of three replicates. The *error bar* represents SD

Table 2: Correlation coefficients between leaf symptom expression and antioxidative enzyme activities under control and iron excess condition

	Treatment	SOD activity	CAT activity	APX activity	GR activity
Leaf symptom expression	1000 mg L^{-1} Fe^{2+}	-0.93*	-0.81*	-0.44*	-0.14*
Leaf symptom expression	2 mg L^{-1} Fe^{2+}	-0.89*	-0.85*	-0.67*	-0.83*

the supperoxides dismutation reaction in reversible direction. In the present investigation, the induction of APX activity is much more in the cultivar Maibetikala as compared with that of the cultivars Lalgura, Katakchara, Kanatara and Chinari. One possible reason could be that sensitive cultivars may transmit a stronger oxidative stress signal in comparison to the tolerant cultivars in response to stress. A similar increase in APX and GR activity in rice leaf was reported in rice leaf in response to excess iron (Stein et al., 2009a).

4. Conclusion

Tolerant rice cultivars have the ability to retain high iron concentration in root tissues. Expression of leaf symptoms depend largely upon varietal susceptibility than leaf iron content. Relative tolerance of cultivars Kanatara, Katakchara, Lalgura and Chinari can also be attributed to its ability to counter the oxidative stress via enhancement of antioxidative enzymatic activities. Based on these findings, it can be concluded that Lalgura and Chinari expressed better tolerance to iron toxicity.

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